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Starvation Treatment Increases Transient Expression Frequency of a Foreign Gene Introduced into *Eustoma grandiflorum* Root Tissues by Particle Bombardment

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Root explants of *Eustoma grandiflorum* were bombarded with plasmid pBI221 harboring the *uidA* gene encoding β-glucuronidase (GUS) driven by a cauliflower mosaic virus 35 S promoter, and the effects of pre- and post-bombardment culture in various starvation media on the number of blue spots of GUS-expressing cells were studied. It was found that pre- and post-bombardment culture in nitrogen-depleted media increased the expression frequency of the transgene, by 4- to 8-times that of the control, and that 3-h pre-culture in the nitrogen-depleted medium gave the highest expression frequency.

Introduction

Transient expression of foreign genes introduced by particle bombardment is influenced by various physical parameters[1,2]. Pre-culture of target tissues or cells in appropriate culture media[3-5], and treatment of target cells with media of high osmotic pressure[6-8] is reported to increase the expression frequency, at most by 10-fold, of the *uidA* gene introduced by particle bombardment. No reports, however, have been published on the effects of starvation treatments on the expression of transgenes introduced by particle bombardment. During our study on the optimization of bombardment conditions and the stable transformation of *Eustoma grandiflorum* using particle bombardment[9], we have found that the treatment of the root explants with nitrogen -depleted medium markedly increased transient expression frequency of the *uidA* gene.

Materials and Methods

1. *Plant materials*

Eustoma grandiflorum cv. Glory White plants were grown in a germination medium (GM), which contains macronutrients (1/2 strength), micronutrients, vitamins, inositol and amino acid of MS medium[10], and 1.5% sucrose (pH 5.8) at 26°C as reported previously[9]. From 15-day-old seedlings, root explants (0.5 to 1 cm long) were cut in a shoot-inducing medium (SIM) or an appropriate starvation medium. SIM contains MS salts, vitamins, inositol, amino acid, 3% sucrose, 1 μM 6-benzylaminopurine and 0.1 μM α-naphthylacetic acid (pH 5.8). The following starvation media were used: SIM lacking both NH_4NO_3 and KNO_3, and supplemented with 1.4 g/l KCl (SIM-N); SIM lacking KH_2PO_4 with K being supplied as 0.094 g/l KCl (SIM-P); SIM without sucrose (SIM

-C); SIM-N lacking KH_2PO_4, and supplemented with 0.094 g/*l* KCl (SIM-NP); SIM-N without sucrose (SIM-NC); and SIM-N lacking both KH_2PO_4 (with K being supplied as 0.094 g/*l* KCl) and sucrose (SIM-NPC).

Root explants (*ca.* 120 root sections or *ca.* 40 mg fresh weight.) were placed so as to form a disk (3 cm diameter) on a filter paper (ADVANTEC TOYO No. 2, 5.5 cm diameter, Toyo Roshi Kaisha Ltd., Tokyo Japan). The filter paper with root explants was then transferred onto a Petri dish (5.8 cm internal diameter) that contained a gellan gum (0.2%) medium whose composition was otherwise the same as the liquid medium in which root explants had been excised. Root explants were then cultured for 24 h (unless otherwise stated) under 15 h light (50 $\mu E\ m^{-2}s^{-1}$) /9 h dark at 26°C. After culture, the filter paper with explants was transferred onto an empty Petri dish, and subjected to bombardment. After being bombarded, the filter paper with explants was then transferred back to the respective medium and incubated for another 24 h, and assayed for GUS expression.

2. *Plasmid DNA*

Plasmid pBI221 (Clontech, Palo Alto, CA), which has the *uidA* gene under the control of the cauliflower mosaic virus 35 S promoter and nopaline synthetase polyadenylation region, was used.

3. *Gene delivery*

Gold particles (1.1 μm in diameter, Tokuriki Honten Co., Tokyo, Japan) were coated[11] with pBI221, and a single shot was given to each target sample using an automatic pneumatic particle gun device (model 260, Rehbock Co., Tokyo) as reported previously[9,12].

4. *GUS assay*

Histochemical GUS enzyme assay[13] was carried out essentially as described elsewhere[14,15]. GUS-expressing cells were detected as blue-colored spots under a binocular microscope. The size of the blue spots varied from 400 to 1,000 μm, but each spot, regardless of its size, was considered as one GUS-expression unit.

Results and Discussion

Fig. 1 shows the number of blue spots of GUS-expressing cells on *Eustoma grandiflorum* root

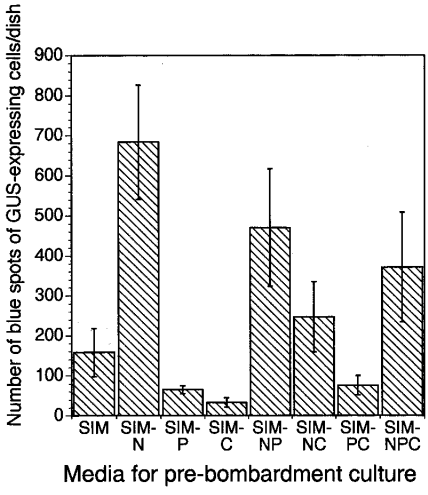

Fig. 1 Effects of various starvation treatment on the number of blue spots of GUS-expressing cells on *Eustoma grandiflorum* root explants.
Eustoma grandiflorum root explants were subjected to pre-bombardment culture on starvation media or on control medium (SIM) for 24 h, and, after being bombarded, to post-bombardment culture on the respective medium for another 24 h. The vertical bar indicates the standard deviation of three replicates.

explants that were subjected to pre-bombardment culture on various starvation media or on control medium (SIM) for 24 h, and, after being bombarded, to post-bombardment culture on the respective medium for another 24 h. Clearly, starvation treatments with media lacking nitrogen sources (SIM-N, SIM-NP, SIM-NC and SIM-NPC) were found to increase the number of blue spots of GUS-expressing cells than the control (SIM). But treatments with other media (SIM-P, SIM-C and SIM-PC) decreased the number of blue spots of GUS-expressing cells. Among all of the N-lacking media, the simplest SIM-N medium was the most effective and the number of blue spots of GUS-expressing cells was more than 4-fold that of the control (see **Fig. 1**). This suggests that depletion of phosphates and/or sucrose is inhibitory (even in the N-lacking condition) to the expression of the transgene introduced in *Eustoma grandiflorum* root explants. A typical photomicrograph of blue spots of GUS-expressing cells on *Eustoma grandiflorum* root explants is shown in **Fig. 2**.

When root explants that had been precultured on SIM-N for 24 h were subjected to post-bombardment culture on the complete SIM, the GUS expression frequency was decreased almost to the level of the control that were pre- and post-cultured on SIM for 24 h as shown in **Fig. 3**. Similarly, when root explants precultured on SIM were subjected to post-bombardment culture on SIM-N, the GUS expression frequency was again similar to that of the control level. These results show that the best result of the expression frequency of the *uidA* gene in *Eustoma grandiflorum* root explants was obtained when both pre- and post-culture were made in nitrogen-depleted medium.

Fig. 4 shows the changes in the number of blue spots of GUS-expressing cells on the root explants that were pre-cultured with SIM (or SIM-N) for 0 to 120 h, bombarded with pBI221, cultured for 24 h on the same medium, and then assayed for GUS expression. The number of blue spots of GUS-expressing cells did not largely change by pre-culture on SIM. However, this number drastically increased by pre-culture on SIM-N, and attained a peak value at 3 h. The peak value was 953± 152 (average of 3 replicates±SD), which was more than 4-fold that of the control. Thereafter, the number gradually decreased and attained almost control level after 72 to 120 h of pre-culture (see **Fig. 4**).

Depletion of nitrogen from culture medium has been reported to activate transcription of specific genes in cultured rice cells[16]. In the present study, the effect of starvation treatment of nitrogen on the *uidA* gene expression under the cauliflower mosaic virus 35 S promoter was observed. Whether or not the effect of depletion of nitrogen from the culture medium on the transient expression of the introduced transgene, observed in the present study, in *Eustoma* root explants is

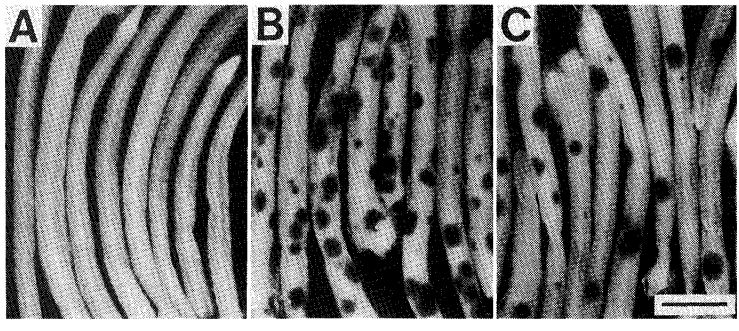

Fig. 2 Typical photomicrographs of blue spots of GUS-expressing cells in root explants of *Eustoma grandiflorum*.
Root explants were bombarded with non-coated gold particles (A) or pBI221-coated gold particles (B and C). Pre- and post-bombardment culture (24 h each) was made with SIM-N (A and B) or with SIM (C), and histochemically-assayed for GUS expression. The bar indicates 0. 5 mm.

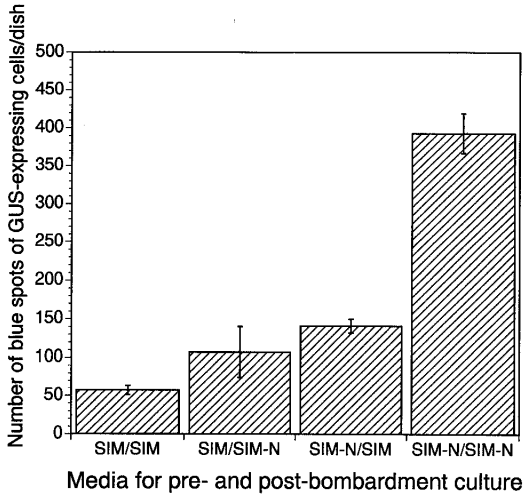

Fig. 3 Effect of nitrogen starvation in the pre- and/or post-bombardment culture on the number of blue spots of GUS-expressing cells in root explants of *Eustoma grandiflorum*.
Both pre- and post-bombardment cultures (24 h each) were made with SIM (SIM/SIM) or with SIM-N (SIM-N/SIM-N), and pre- and post-bombardment culture was made respectively with SIM and with SIM-N (SIM/SIM-N) and vice versa (SIM-N/SIM). The vertical bar indicates the standard deviation of three replicates.

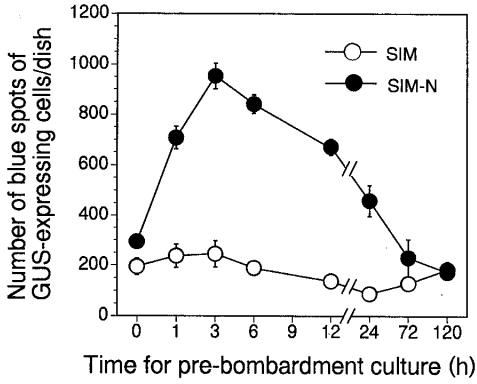

Fig. 4 Changes in the number of blue spots of GUS-expressing cells in root explants of *Eustoma grandiflorum* as a function of the time for pre-bombardment culture with SIM or with SIM-N.
Root explants were precultured for 0 to 120 h with SIM or SIM-N and bombarded with pBI221, followed by culture with the same medium for 24 h and histochemical GUS assay. Root explants were cut in GM for the pre-culture treatment of 0 h. The vertical bar indicates the standard deviation of three replicates.

attributable to transcription and/or translation of the introduced transgene is a subject of future study.

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《和文要約》

飢餓処理がパーティクルガンによりトルコギキョウ根組織に導入されたトランスジーンの一過的遺伝子発現効率を上昇させる

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発芽15日目のトルコギキョウ根組織から切り出した外植片を，茎葉誘導培地(SIM)およびSIMから窒素，リン，炭素源を除いた培地(SIM-N, -P, -C, -NP, -NC, -NPC)で24時間培養(前培養)し，プラスミドpBI221(CaMV 35 Sプロモーター，GUS遺伝子を持つ)をパーティクルガンにより導入した．その後，前培養と同一培地で24時間培養(後培養)し，GUS遺伝子の一過的発現を組織化学的に解析した．その結果，GUS遺伝子の発現は，前後培養をN欠培地(SIM-N, -NP, -NPC)で行ったときに高い値を示し，SIM-Nで最も高い値が得られた(コントロールの4倍以上)．このN欠の効果は，前培養あるいは後培養のみでは見られず，培養の前後ともN欠である必要があった．また，3時間のN欠前培養で最も高い発現が認められ，コントロールの4倍以上であった．